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Experimental and Clinical Studies on Plasma Leptin in Obese Dogs

Katsumi ISHIOKA[1)], Mohamed M. SOLIMAN[1)], Mayumi SAGAWA[2)], Fumio NAKADOMO[3)], Haruki SHIBATA[4)], Tsutomu HONJOH[4)], Akira HASHIMOTO[5)], Hiroshi KITAMURA[1)], Kazuhiro KIMURA[1)] and Masayuki SAITO[1)]*

[1)]Laboratory of Biochemistry, Department of Biomedical Sciences, and [5)]Laboratory of Pathobiology, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, [2)]Nippon Pet Food Co., Ltd. Research Center, 2020 Umeyama, Asaba-cho, Iwata 437–1105, [3)]Osaka Prefectural College of Nursing, 3–7–30, Habikino 583–8555 and [4)]Morinaga Institute of Biological Science, 2–1–1 Shimosueyoshi 2, Tsurumi, Yokohama 230–8504, Japan

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ABSTRACT. Leptin is a protein synthesized and secreted primarily by adipocytes, and the circulating leptin concentration is elevated in obese humans and rodents. Recently, we have established a sandwich enzyme-linked immunosorbent assay for canine leptin. In the present study, plasma leptin concentrations were measured in experimentally developed obese beagles and in clinically obese dogs. When 5 male beagles were given a high-energy diet for 3 months, all of them became obese and the plasma leptin concentration significantly increased from 2.4 ± 1.2 to 4.9 ± 0.9 *n*g/m*l*, positively correlating with body fat content estimated by the deuterium oxide dilution method (r=0.87). The leptin concentrations of plasma samples collected from 59 dogs in veterinary practices were compared with their body condition scores (BCS). The plasma leptin concentrations of obese dogs were 9.7 ± 0.7 and 12.3 ± 1.5 *n*g/m*l* at BCS=4 and BCS=5, respectively, which were significantly higher than those of optimal (BCS=3) dogs (2.7 ± 0.3 *n*g/m*l*). There was no significant effect of sex and breed. A weak positive correlation (r=0.37) was found between the plasma leptin concentration and age, probably due to the lesser content of visceral fat in puppies younger than 1 year old. These results indicate that plasma leptin is a good index of adiposity in dogs regardless of breed, age and sex, and may be useful for quantitative assessment of obesity in small animal practice.
KEY WORDS: canine, ELISA, leptin, obesity.

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Obesity is the most common nutritional disorder in small animal medicine, and is associated with other various diseases and/or risks, such as diabetes, pancreatitis, cardiovascular diseases, articular problems and increased surgical risk [4, 10]. One of the major constraints for the study of obesity in companion animals is the absence of the simple and reliable methods for accurate estimation of adiposity and diagnosis of obesity [13]. For example, the body condition score (BCS) is widely used as a diagnostic scale of obesity in dogs, but this is a somewhat subjective estimate [9]. Alternatively, ultrasonographic examination of subcutaneous fat thickness was reported to be useful for quantitative estimation of body fat mass, but this is not easy for routine use [1, 20].

Leptin, the product of the *ob* gene, is a protein synthesized and secreted primarily by adipose tissue [21]. In humans and rodents, the blood leptin concentration is known to positively correlate with body fat content and to be higher in obesity [11]. Recently, we cloned canine leptin cDNA, produced recombinant canine leptin in *Escherichia coli* [6], and established a sandwich enzyme-linked immunosorbent assay (ELISA) for canine leptin [7]. Using this canine-specific ELISA method, we assayed plasma leptin of 20 female beagles with a wide range of body fat contents, and found a highly positive correlation between the plasma leptin concentration and body fat content [16]. These results suggest that plasma leptin is a quantitative marker of adiposity and obesity in dogs, as in humans and rodents. To extend and confirm this idea, in the present study, we compared plasma leptin concentrations in male beagles before and after the development of obesity, and also in clinical cases of various breeds, ages and sexes with the BCS.

* CORRESPONDENCE TO: SAITO, M., Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan.

MATERIALS AND METHODS

Plasma leptin in experimentally developed obese beagles: Five male beagles (3 years old, 11.5–13.3 kg) were housed in a climate-controlled room and fed a standard dry diet (FIELD, Petline, Tokyo) once daily at 10:00. Water was available *ad libitum*. Body fat weight was estimated by the isotopic dilution method as described previously [3, 15]. Briefly, after 16 hr fasting, water was withheld from the dogs and they were injected with deuterium oxide (99.8% D_2O, Merck, Darmstadt, Germany) into the cephalic vein at a dose of 0.2 g/kg body weight. Before and 90 min after the injection, blood (8–12 m*l*) was collected from the jugular vein into heparinized tubes. The plasma samples (4 m*l*) were purified by vacuum sublimation, and the concentration of deuterium was measured using a gas-choromatography thermal conductivity detection system (Automatic Deuterium Oxide Analyzer HK-102, Shoko Co., Ltd., Tokyo). Body fat content was calculated by putting the results into the validated equation proposed by Burkholder and Thatcher [3], and expressed as percentage of body weight. Plasma samples obtained before D_2O injection was stored at –80°C for leptin assay. These dogs were then fed a high-energy diet (8400 kJ/day, p/d dry, Hill's Pet Nutrition, Inc.,

Topeka, KS, U.S.A.). After 3 months, body fat content was estimated as previously. Animal care and procedures were in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University.

Plasma leptin in clinical cases: Plasma samples were collected from 59 dogs visiting 4 veterinary practices in Japan. The name, breed, age, sex, body weight, feeding condition, and present illnesses of individual dogs were confirmed from a record sheet kept by each practice. They were summarized as follows: male/female, 28/31; age, 5 months-16 years old; breed/number, Beagle/3 , Bichon Frise/1 , Cocker Spaniel/1, Golden Retriever/4, Maltese/2, Miniature Dachshunds/7, Miniature Schnauzer/1, Pekingese/2, Pomeranian/1, Shetland Sheepdog/3, Shiba Inu/3, Shih Tzu/6, Siberian Husky/1, Tosa Inu/1, Yorkshire Terrier/1, Welsh Corgi/2, and mixed breeds/20. The BCS was assessed and expressed as a five point scale: 1, thin; 2, lean; 3, optimal; 4, obese; 5, gross. In some cases the BCS was determined by comparing the body weight with the ideal body weight of the corresponding breed according to a previously reported table (less than 85%, BCS=1; 86–94%, BCS=2; 95–106%, BCS=3; 107–122%, BCS=4; more than 123%, BCS=5) [17]. Plasma samples were taken from dogs without clinical problems after overnight fasting, and stored at –80°C for 1–6 months until leptin assay.

Sandwich ELISA for canine leptin: The plasma leptin concentration was measured by the previously reported method of sandwich ELISA using an anti-canine leptin antibody [7] with minor modifications. Briefly, each well of a 96-well microplate was coated with 0.2 μg of the purified rabbit anti-canine leptin antibody and incubated for 2 hr at room temperature. After washing the wells twice with TBS-T (10 mM Tris-HCl, pH 7.4/150 mM NaCl/0.05% Tween 20), 300 μl of TBS-T-BSA (TBS-T/0.1% bovine serum albumin) was applied to each well for blocking. After 2 hr incubation, the wells were washed twice with TBS-T, and incubated overnight at 4°C with 80 μl of 30% normal rabbit serum and either 20 μl of the plasma sample or recombinant canine leptin (0.5–32 *n*g/m*l*). Then the wells were washed 5 times with TBS-T, and incubated with 100 μl of horseradish peroxidase-conjugated anti-canine leptin antibody (0.2 μg/ml) for 4 hr at 4°C. After washing 7 times, the wells were incubated with 100 μl of a peroxidase substrate solution in the dark for 30 min at room temperature. After stopping the reaction by adding 50 μl of 1N H_2SO_4, the absorbance of each well was measured at 450 nm.

Statistical analyses: Linear regression and correlation analyses were conducted with a computer program (Statview 5.0, SAS Institute Inc, Cary, NC, U.S.A.) for the plasma leptin concentration to body weight, fat content, and age. Statistical comparisons of the clinical cases were performed by analysis of variance with Scheffe's test.

RESULTS AND DISCUSSION

Plasma leptin in experimentally developed obese beagles: In a previous study, we assayed plasma leptin of 20 spayed female beagles with a wide range of body fat contents, and found a highly positive correlation between the plasma leptin concentration and body fat content [16]. In the present study, we compared plasma leptin concentrations in beagles before and after the development of obesity. For this, 5 male beagles were fed a high-energy diet for 3 months, during which body weight increased in all individuals (Table 1). To estimate body fat content, we used the D_2O dilution method, which is known as the most reliable and non-invasive method in various species, including dogs. During the 3-month period, body fat content increased similarly to body weight, but much more remarkably (1.6–2.4-fold). Thus, feeding the high-energy diet effectively produced obesity in beagles.

The plasma leptin concentration was measured using the canine-specific ELISA method. We have found that the plasma leptin concentration decreases gradually after prolonged starvation but increases remarkably 6–12 hr after food intake (unpublished observations). To minimize the effects of feeding and fasting, all blood samples were taken after 16-hr fasting. As shown in Table 1, although the plasma leptin concentration was considerably different among the 5 beagles, it increased in all individuals after the 3-month period.

To clarify the relations of the plasma leptin concentration to body weight and body fat content, 10 data obtained from the 5 beagles before and after the 3-month period were pooled and subjected to linear regression and correlation analyses. As shown in Fig. 1, no significant correlation was found between the plasma leptin concentration and body weight (r=0.54, p=0.11). However, a highly positive corre-

Table 1. Body weight, body fat content, and plasma leptin concentration of 5 beagles, before and after 3-month feeding of a high-energy diet

Beagle No.	Body weight (kg)		Body fat (%)		Leptin (*n*g/m*l*)	
	Before	After	Before	After	Before	After
1	12.7	14.1	22.0	42.6	6.5	7.2
2	11.5	14.3	14.4	31.4	1.7	4.2
3	12.9	13.8	18.3	29.8	3.0	5.1
4	11.9	14.1	8.5	18.3	0.1	1.8
5	13.3	15.1	15.3	36.9	0.5	6.1
Mean ± SE	12.5 ± 0.3	14.3 ± 0.2**	15.7 ± 2.2	31.8 ± 4.1**	2.4 ± 1.2	4.9 ± 0.9*

*, p<0.05; **, p<0.01, vs Before.

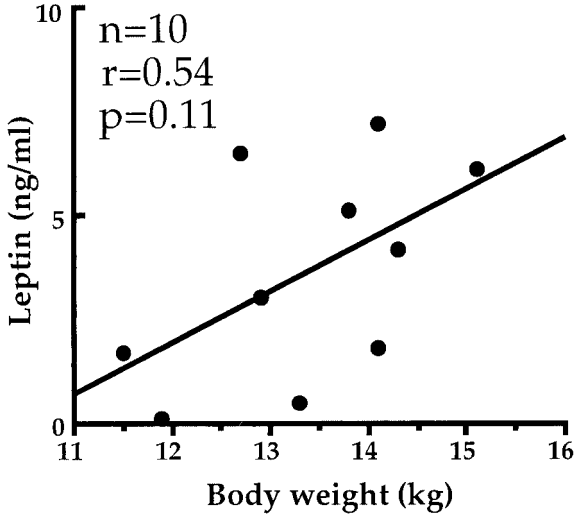

Fig. 1. Correlation between plasma leptin concentration and body weight. Linear regression and correlation analyses were performed for the data in Table 1.

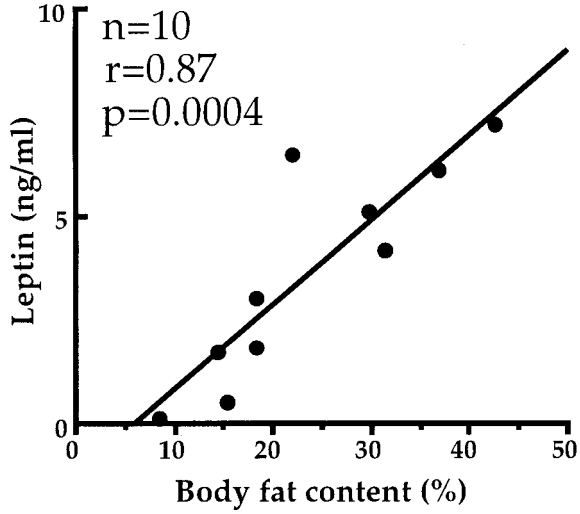

Fig. 2. Correlation between plasma leptin concentration and body fat content. Linear regression and correlation analyses were performed for the data in Table 1.

lation of the plasma leptin concentration was found to body fat content (r=0.87, p=0.0004, Fig. 2). In our previous study using 20 lean and obese beagles, we found that the plasma leptin concentration correlated well with not only body fat content (r=0.920) but also body weight (r=0.856) [16]. Despite this discrepancy, our results clearly showed that plasma leptin reflects body fat content more than body weight, and thereby is a good biochemical index of adiposity/obesity in beagles. This is quite understandable because leptin is synthesized exclusively in adipose tissue, at least as far as known, in beagles.

Plasma leptin in clinically obese cases: In the above studies using beagles, the subjects were chosen for homogeneity in their age, sex, breed, and housing conditions such as room temperature, lighting conditions and feeding time. In small animal practices, however, the subjects and conditions are heterogeneous, especially the somatoscopic parameters among the breeds. To examine whether the positive relationship between plasma leptin and adiposity seen in beagles is also the case in these clinical cases, we measured plasma leptin concentrations of apparently obese dogs visiting 4 veterinary practices and compared them with those of non-obese dogs. As in the above studies using beagles, blood samples were collected after overnight fasting. Since the plasma leptin concentration is also influenced by insulin and glucocorticoids (unpublished observations), the samples from the dogs treated with these hormones were also eliminated. Thus, we obtained blood samples from a total of 59 dogs, consisting of 17 breeds, 28 males and 31 females, and aged 5 months to 16 years.

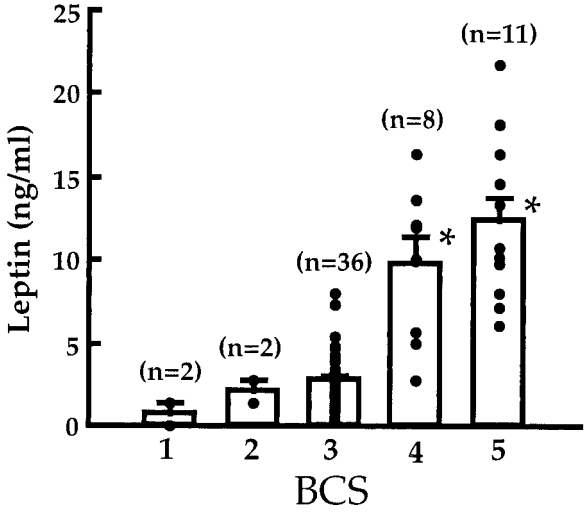

Fig. 3. Plasma leptin concentration of dogs visiting veterinary practices. Fifty-nine dogs were divided to 5 groups depending on their body condition scores (BCS). The values are presented as mean±SE (columns) with individual values (dots). *, p<0.0001 vs BCS=3.

Plasma leptin concentrations were considerably different among individual dogs, ranging from 0 (undetectable) to 21.7 *n*g/m*l*. There was no noticeable difference or tendency among the breeds. To examine the possible relation to adiposity and obesity, we divided the dogs into 5 groups according to the BCS, a widely used index of nutritional conditions and obesity in small animal practice. Of the 59 dogs, 36 were judged optimal (BCS=3), while 19 were obese (BCS>= 4). Only 4 dogs were lean (BCS=< 2). As summarized in Fig. 3, the mean leptin concentration of the optimal dogs was 2.7 ± 0.3 *n*g/m*l*, which was in the same range as in beagles ([7] and Table 1), and also in non-obese humans and rodents [11]. The plasma leptin concentration of obese dogs was 3–4-fold higher than that of optimal dogs, being 9.7 ± 0.7 *n*g/m*l* at BCS=4 (p<0.0001, vs BCS=3) and

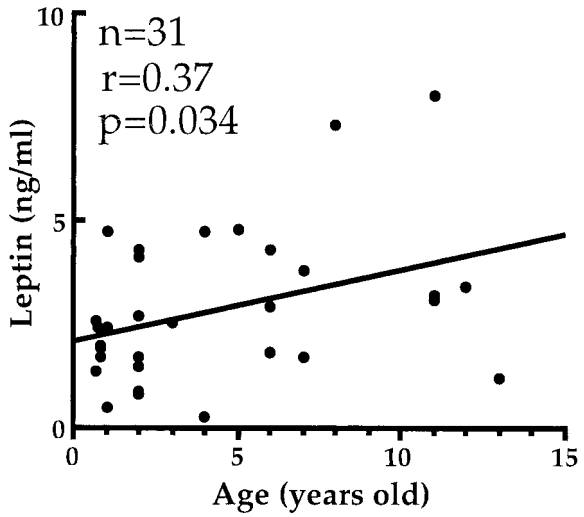

Fig. 4. Correlation between plasma leptin concentration and age. Linear regression and correlation analyses were performed for the values of 31 optimal dogs.

12.3 ± 1.5 *n*g/m*l* at BCS=5 ($p<0.0001$, vs BCS=3). In contrast, plasma leptin of lean dogs tended to be lower than in optimal dogs, although the difference was not significant because the number of dogs was too small for statistical analysis.

It is to be noted that plasma leptin concentrations were considerably different among individual dogs with the same BCS. This may be due to, at least in part, the different times after food intake: that is, although blood samples were collected after overnight fasting, the fasting period could not be controlled in such clinical cases as strictly as in the above studies using beagles. Alternatively, BCS is a somewhat subjective estimate, and may not always correlate to body fat content. In fact, it is sometimes difficult to discriminate, for example, BCS=4 from BCS=3. Despite these variations, the present results revealed significantly higher plasma leptin concentrations in dogs with higher BCS.

These results seem quite consistent with those reported by Kitagawa *et al.* [8], who measured plasma leptin immunoreactivity of 28 lean and 45 obese patient dogs using human leptin as a standard. Although in their studies the values were relative to human leptin and the possible influences of feeding-fasting cycles were not considered, a 2.4-fold higher level of plasma immunoreactive leptin was observed in obese dogs. From these results, together with those using beagles, it can be concluded that plasma leptin levels are positively correlated to adiposity and higher in obese dogs regardless of their breed, age and sex. In other words, plasma leptin may be useful for quantitative diagnosis of adiposity/obesity of dogs in small animal practice.

The possible relation of plasma leptin to sex was also examined in the 36 dogs with a BCS of 3. The mean concentration of plasma leptin tended to be higher in females (3.0 ± 0.4 *n*g/m*l*, n=18) than in males (2.3 ± 0.4 *n*g/m*l*, n=18), although the difference was not significant. The plasma leptin concentration is known to be higher in female humans [14] and mice [5]. The reasons for such sex differences are not clear, but one may non-adipose tissue production of leptin. In fact, it has been reported that leptin is synthesized in the human placenta [12] and in the rat stomach [2]. We confirmed previously that leptin mRNA was detected only in adipose tissues of adult beagles and not in 10 other organs [6], but we have not examined the placenta and stomach. The effects of age on the plasma leptin concentration were also examined in the same 36 dogs. As shown in Fig. 4, a weak, but significant correlation between the plasma leptin concentration and age was found ($r=0.37$, $p=0.034$). This seems compatible to a report that the plasma leptin concentration is lower in younger animals before sexual maturation [18]. It is also known in dogs that visceral fat content is lower in sexually immature puppies [13, 19]. Since the visceral fat content is difficult to evaluate by physical examination, the true fat content may be less than that expected from the BCS in puppies. Collectively, it is possible that the significant correlation may be due to a lesser content of visceral fat in younger subjects. In fact, there was no significant effect of age in dogs older than 1 year old.

In conclusion, plasma leptin is a biochemical index of adiposity in dogs as in other species. Since plasma leptin is elevated in obese dogs regardless of breed, age and sex, it should be useful for quantitative assessment of obesity in small animal practice. It should also be noted that the assay method is simple and non-invasive compared with other quantitative methods such as ultrasonography and the D_2O dilution method.

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